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Rapid report

Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling

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Key words: abscisic acid (ABA), drought, guard cells, hydrogen sulphide (H_2S), stomatal closure.

Summary

- Hydrogen sulphide (H_2S) has been proposed as the third gasotransmitter. In animal cells, H_2S has been implicated in several physiological processes. H_2S is endogenously synthesized in both animals and plants by enzymes with L-Cys desulphydrase activity in the conversion of L-Cys to H_2S, pyruvate and ammonia.
- The participation of H_2S in both stomatal movement regulation and abscisic acid (ABA)-dependent induction of stomatal closure was studied in epidermal strips of three plant species (*Vicia faba*, *Arabidopsis thaliana* and *Impatiens walleriana*). The effect of H_2S on stomatal movement was contrasted with leaf relative water content (RWC) measurements of whole plants subjected to water stress.
- In this work we report that exogenous H_2S induces stomatal closure and this effect is impaired by the ATP-binding cassette (ABC) transporter inhibitor glibenclamide; scavenging H_2S or inhibition of the enzyme responsible for endogenous H_2S synthesis partially blocks ABA-dependent stomatal closure; and H_2S treatment increases RWC and protects plants against drought stress.
- Our results indicate that H_2S induces stomatal closure and participates in ABA-dependent signalling, possibly through the regulation of ABC transporters in guard cells.

Introduction

Hydrogen sulphide (H_2S) is a small gas with a characteristic odour of rotten eggs. In aqueous solutions and at physiological pH, two-thirds of the H_2S content is dissociated in the species HS^- or S^{2-}; however, the amount of S^{2-} released is negligible (Beauchamp *et al.*, 1984). The solubility of H_2S in lipophilic solvents is fivefold greater than in water (Wang, 2002). Studies on nitric oxide (NO) and carbon monoxide (CO) have highlighted the relevance of gaseous signalling molecules in biology. Further evidence suggests that an additional endogenous gasotransmitter, H_2S, plays physiological functions as important as NO and CO in animal systems (Wang, 2002; Yang *et al.*, 2008). Anti-inflammatory, vasorelaxant and neuroprotective functions for H_2S in mammals have already been described, among others (Li & Moore, 2008; Yang *et al.*, 2008). Additionally, H_2S has been reported to participate in different physiological processes such as smooth muscle relaxation, neuronal excitability and blood pressure regulation (Wang, 2002). In mammals, most of the endogenously synthesized H_2S occurs via two pyridoxal-5′-phosphate-dependent enzymes. Cystathionine β-synthase (CBS, EC 4.2.1.22) hydrolyses L-cysteine to L-serine; and cystathionine γ lyase (CSE, EC 4.4.1.1) hydrolyses L-cysteine to produce H_2S, pyruvate and ammonia (Wang, 2002; Qu *et al.*, 2008). Both enzymes participate in cysteine

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metabolism, where CSE acts as a L-Cys desulphydrase. In plants, H_2S is generated endogenously by the L-Cys desulphydrase DES1 (E.C. 4.4.1.1), recently characterized in *Arabidopsis thaliana* (Alvarez *et al.*, 2010). Moreover, L-Cys desulphydrase activity was previously reported as L-CDES for other plant species (Papenbrock *et al.*, 2007). In addition, the widely studied cysteine synthesis complex (CSC) consumes H_2S during the synthesis of L-Cys from *O*-acetyl serine (OAS) which is catalysed by the enzyme *O*-acetyl(thiol)serinelyase (OAS-TL) (Wirtz & Hell, 2006). In the last 2 yr, researchers have reported the protective effect of H_2S counteracting oxidative stress in plants (Zhang *et al.*, 2008, 2009a, 2010). H_2S was also involved in root organogenesis (Zhang *et al.*, 2009b). Despite the advances described earlier, knowledge of the mechanisms of action and biology of H_2S as a signalling molecule in plant systems is still limited.

In plants, NO and CO are well established messenger molecules and inducers of physiological changes in guard cells leading to stomatal closure (Garcia-Mata & Lamattina, 2001; Neill *et al.*, 2002; She & Song, 2008). Stomata are pores of plant aerial tissues conformed by a pair of guard cells. These specialized cells receive and integrate a great number of external and internal stimuli to accurately respond to plant physiological requirements. Among all the stimuli sensed by guard cells, the phytohormone abscisic acid (ABA) is by far the most studied. ABA regulation of stomatal movement has become a model system for the study of signalling processes in plants. Ion channels, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and intracellular pH regulation are well established components of ABA signalling in guard cells. In animal cells, it has also been proven that H_2S exerts its biological action through the regulation of ion channel activity, modulation of $[Ca^{2+}]_{cyt}$ and intracellular pH, as well as in other ways (Lee *et al.*, 2006, 2007; Tan *et al.*, 2010). We were therefore interested to study the functionality of H_2S in a well-characterized plant model system such as stomatal movement. In this report we present evidence supporting a role of H_2S in plants as a novel component of guard cell signalling in ABA-induced stomatal closure. The potential of H_2S to enhance plant tolerance to water deficit conditions appears to be relevant and will be discussed.

Materials and Methods

Plant material

Vicia faba (L.) var. major and *Impatiens walleriana* Hook. f. were grown in soil : vermiculite (3 : 1, v/v) at 25°C with 16 : 8 h light : dark cycles. *Arabidopsis thaliana* (L.) Heynh ecotype Columbia was grown in soil : perlite : vermiculite (1 : 1 : 1, v/v) at 25°C and with a 16 : 8 h light : dark photoperiod and watered with *Arabidopsis thaliana* salts (ATS) (Wilson *et al.*, 1990) nutritive medium.

Chemicals and treatments

Sodium hydrosulphide (NaHS), DL-propargylglicine, glibenclamide (Gli) and hypotaurine were purchased from Sigma, and *p*-(methoxyphenyl)morpholino-phosphinodithioic acid (GYY 4137) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Stomatal aperture treatments were performed on excised epidermal strips. Immediately after striping, epidermal peels were floated in opening buffer (10 mM K-MES, pH 6.1, 10 mM KCl) for 2 h (*V. faba* and *I. walleriana*) or 3 h (*A. thaliana*). Strips were then kept in the same opening buffer and exposed to different treatments. After 90 min, stomata were digitized using a Nikon DS-Fi1 camera coupled to a Nikon Eclipse Ti (Nikon, Tokyo, Japan). The stomatal aperture was measured using IMAGEJ analysis software (NIH, Bethesda, MD, USA).

Relative water content (RWC) was measured according to Garcia-Mata & Lamattina (2001). *Impatiens* plants were watered with 100 ml of NaHS for 24 h and then watering was suspended for 4 d. RWC was measured in three different leaves from three different plants in at least three independent experiments. *V. faba* plants were watered with 100 ml of NaHS for 24 h, then leaves from five different plants were cut and placed on a white paper under light at 25°C for 1–5 h.

Viability test

Vicia faba epidermal strips were pretreated for 2 h in opening buffer and 90 min with the different treatments. Strips were then incubated with 5 μM fluorescein diacetate (FDA) for 5 min. FDA is a nonfluorescent compound that is converted to the green fluorescent fluorescein by the activity of nonspecific intracellular esterases. After FDA loading, strips were washed three times with fresh opening buffer and mounted for microscopy. Fluorescence pictures were obtained with a Nikon DS-Fi1 digital camera coupled to an epifluorescence Nikon Ti microscope.

H_2S measurements

Vicia faba and *A. thaliana* leaves were ground with liquid nitrogen to a fine powder and suspended in bidistilled water. After vortexing for 1 min, H_2S was measured for 20 min using a Micro Sulfide Ion Electrode (LIS-146AGSCM; Lazar Research Lab. Inc., Los Angeles, CA, USA) at 25°C. Concentrations of H_2S were determined from a calibration curve made with H_2S donors. Each measurement was repeated in at least three independent experiments.

Statistical analysis

All data were taken from at least three independent experiments. Different treatments were tested using Student's *t*-test

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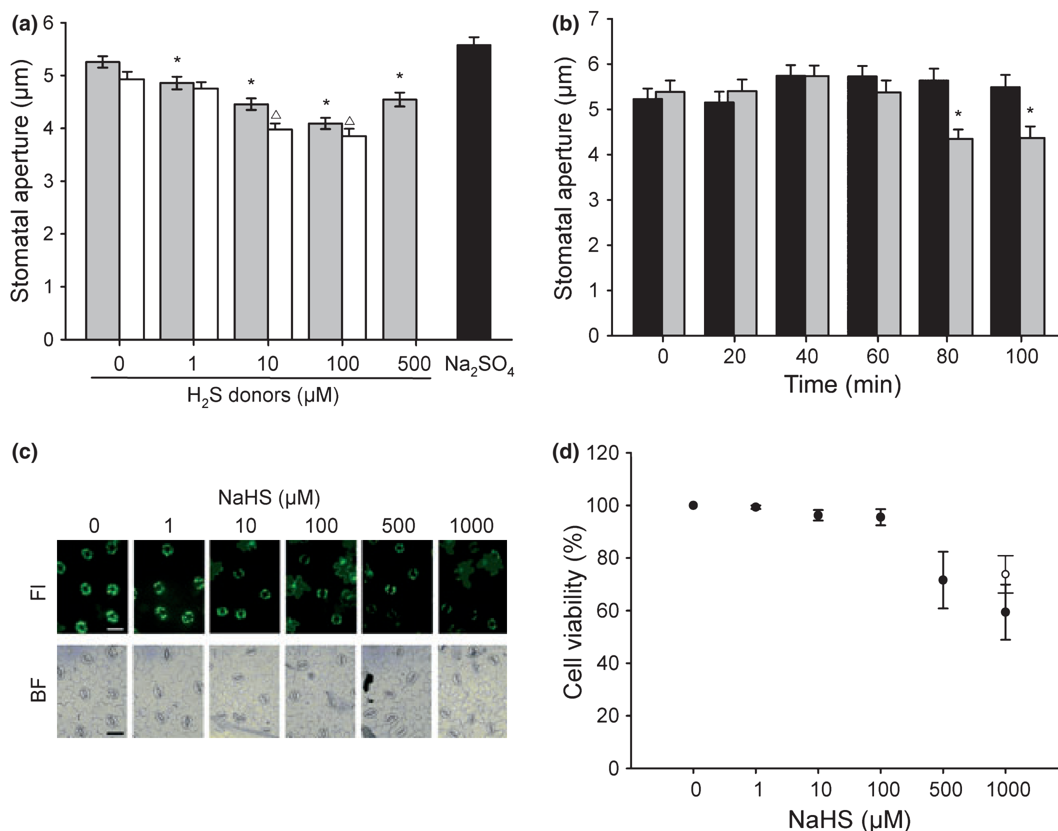

Fig. 1 Hydrogen sulphide (H_2S) induces stomatal closure in a dose-dependent manner. Stomatal aperture measurements were performed on *Vicia faba* epidermal strips preincubated for 2 h in opening buffer (10 mM K-MES, pH 6.1; 10 mM KCl) under light, and treated with different concentrations of the H_2S donors NaHS (grey bars) and GYY 4137 (white bars), or with 500 μM Na_2SO_4 (black bar) for 90 min under light (a) and 100 μM of NaHS (grey bars) or H_2O (black bars) for 100 min (b). Stomatal aperture was quantified every 20 min. Values are expressed as means ± SE. Symbols denote statistical differences with respect to untreated epidermal strips (*t*-test, $P < 0.001$). (c) Viability assay of *V. faba* guard cells. Epidermal strips were treated with different concentrations of NaHS for 90 min and then loaded with 5 μM fluorescein diacetate for 5 min. Images obtained with a Nikon Ti epifluorescence microscope (Ex 585/Em 515–545) depict one representative picture from three independent experiments. (d) Cell viability was quantified by counting the percentage of fluorescent guard cells relative to total guard cells in the bright field after the NaHS treatment (closed circles) and after the NaHS treatment plus 1 h of washing with fresh buffer (open circle). Fl, fluorescence; BF, bright field. Bar, 25 μm.

or Dunn's test, as indicated in the legends to figures using SIGMAPLOT 11 (Systat Software, Inc., Chicago, IL, USA).

Results

To test whether H_2S has any effect on the regulation of stomatal closure, epidermal peels from *V. faba* leaves were treated with different concentrations of the widely used H_2S donor NaHS, ranging from 0 to 500 μM. Fig. 1(a) shows that the H_2S donor induced stomatal closure in a dose-dependent manner, reaching the maximum effect at 100 μM NaHS, while higher concentrations of the donor are less effective. To test the specificity of the response of guard cells to H_2S, epidermal strips were treated with a sulphate sodium salt (Na_2SO_4) that does not release H_2S. Na_2SO_4-treated strips showed stomatal aperture values comparable to those obtained in nontreated strips, supporting the idea that the effect of NaHS was the result of the released H_2S and not the dissociation of the sodium salt or any osmotic effect (Fig. 1a). To confirm the effect of H_2S on stomatal closure, the epidermal strips were treated with another H_2S donor, GYY 4137 (Li *et al.*, 2008). Fig. 1(a) shows the same pattern of stomatal closure for both H_2S donors NaHS and GYY 4137. This last result rules out the effect of any by-product of the donor molecules and confirms the role of H_2S in stomatal closure induction. To test if the incubation time used in Fig. 1(a) lies within the time required to obtain a complete response from H_2S, we assayed a time course experiment measuring stomatal pore size every 20 min after NaHS treatment. Fig. 1(b) shows that the H_2S donor induced a full response at incubation times of 80 min or longer. NaHS concentrations currently used in different animal and plant systems range from 10^{-6} to 10^{-3} M (Doeller *et al.*, 2005; Szabó, 2007; Zhang *et al.*, 2008, 2009a, 2010). Given that no data are available in relation to NaHS treatments of guard cells, we investigated

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whether the concentrations used in Fig. 1(a) could be toxic to guard cells. With that aim, epidermal peels were treated with different concentrations of NaHS and then loaded with 5 μM FDA. Fig. 1(c) shows that NaHS starts to be toxic for *V. faba* guard cells at concentrations ≥ 500 μM. This last result could explain the suboptimal effect of 500 μM NaHS on stomatal closure induction. Furthermore, washing the strips treated with 1 mM NaHS with fresh opening buffer showed only a marginal recovery of the viability percentage (Fig. 1d).

As stated earlier, both NO and CO gases participate in ABA-dependent stomatal closure (Garcia-Mata & Lamattina, 2002; Neill *et al.*, 2002; Cao *et al.*, 2007). To assess whether endogenous H_2S also participates in ABA-mediated induction of stomatal closure, epidermal strips from *V. faba* were pretreated with hypotaurine (HT), which reacts directly with sulphide to form thiotaurine (ThT) (Ortega *et al.*, 2008). Fig. 2(a) shows that NaHS-induced stomatal closure was fully blocked by 200 μM HT, confirming the H_2S scavenger effect of HT. Interestingly, HT pretreatment partially blocked ABA-dependent stomatal closure (Fig. 2a), indicating that H_2S might be involved in ABA signalling, leading to stomatal closure. To confirm endogenous H_2S participation in stomatal closure, epidermal strips were pretreated with DL-propargylglycine (PAG), which inhibits both enzymes, CSE/L-CDES, involved in H_2S biosynthesis (Clausen *et al.*, 1999; Steegborn *et al.*, 1999; see Supporting Information, Fig. S1). Fig. 2(b) shows that ABA-dependent stomatal closure was partially blocked when strips were pretreated with 1 mM PAG (3.85 ± 0.12 and 4.69 ± 0.13 μm for ABA – PAG and ABA + PAG, respectively). The addition of exogenous H_2S (as 100 μM NaHS) to the ABA + PAG treatment restored the stomatal closure to values similar to those obtained from ABA treatment alone, indicating that the reduced response of ABA treatment in the presence of PAG might be the result of the decrease of endogenous H_2S production in guard cells. In addition, NaHS induced stomatal closure regardless of the presence of PAG (Fig. 2b). The same treatments were performed in epidermal peels of the model plant *A. thaliana*, and we confirmed that NaHS also induced stomatal closure in *A. thaliana*, and that in Arabidopsis, PAG has the same effect observed in *V. faba* (Figs S2, Fig. 2b).

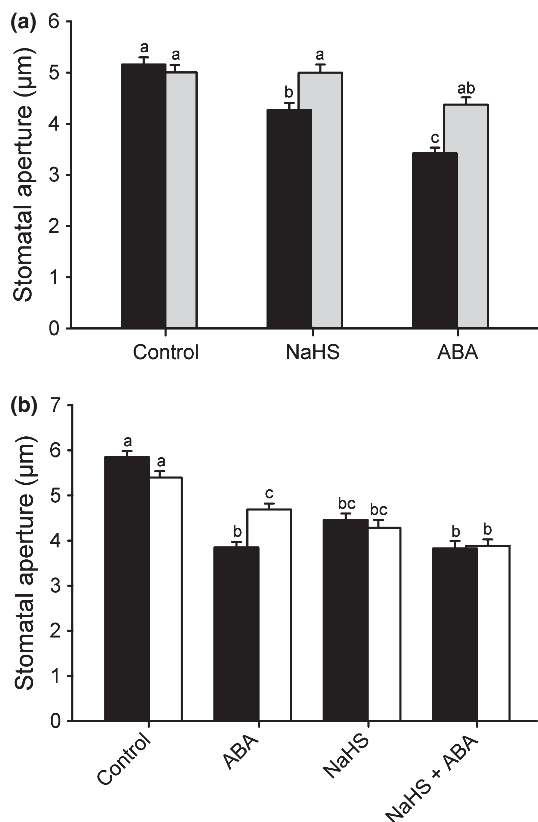

Fig. 2 Hydrogen sulphide (H_2S) is involved in abscisic acid (ABA)-dependent stomatal closure in *Vicia faba*. Epidermal strips were preincubated for 2 h in opening buffer (10 mM K-MES, pH 6.1; 10 mM KCl) in the presence (grey bars) or absence (black bars) of 200 μM hypotaurine (HT) and then treated with 25 μM ABA or 100 μM NaHS (a) or with 1 mM DL-propargylglycine (PAG, white bars) under light, and then treated for 90 min with 25 μM ABA or 100 μM NaHS, or ABA + NaHS (without PAG, black bars) (b). Different letters indicate statistical differences between treatments (Dunn's test $P < 0.001$).

In mammalian systems, H_2S regulates blood vessel calibre by activating K^+-ATP channels (Zhao *et al.*, 2001). The active form of the K^+-ATP channel is a complex of two proteins: a sulphonylurea receptor (SUR: SUR1, SUR2) and an inwardly rectifying K^+ channel, Kir6.2 (Gribble *et al.*, 1997; Babenko *et al.*, 2000). SUR1 is a multidrug resistant protein (MRP) that belongs to the ATP-binding cassette (ABC) transporter family. In Arabidopsis, the MRPs are the best characterized ABC transporters (Martinoia *et al.*, 2002). Among them, AtMRP5 has a high homology with SUR proteins and was shown to bind to the well-established blocker of sulphonylurea receptors, Gli (Leonhardt *et al.*, 1997; Gribble *et al.*, 1998; Martinoia *et al.*, 2002). Interestingly, guard cells from the T-DNA insertion mutant *atmrp5-1* have a partially impaired response to ABA and Ca^{2+} (Klein *et al.*, 2003). More recently, it was proposed that AtMRP5 functions as a Ca^{2+} and anion channel regulator in the plasma membrane of guard cells (Suh *et al.*, 2007). To study the effect of Gli on ABA- and H_2S-induced stomatal closure, *V. faba* epidermal strips were treated with ABA or the H_2S donor NaHS in the presence or absence of Gli. As expected, 1 μM Gli partially blocked ABA-dependent stomatal closure (Fig. 3). Moreover, 1 μM Gli was also sufficient to impair H_2S-induced stomatal closure (3.4 ± 0.09 and 4.79 ± 0.13 μm, respectively, for NaHS – Gli and NaHS + Gli) (Fig. 3). All together, these results suggest that SURs on guard cells might be targets for both H_2S and Gli, and are downstream of ABA-triggered stomatal closure.

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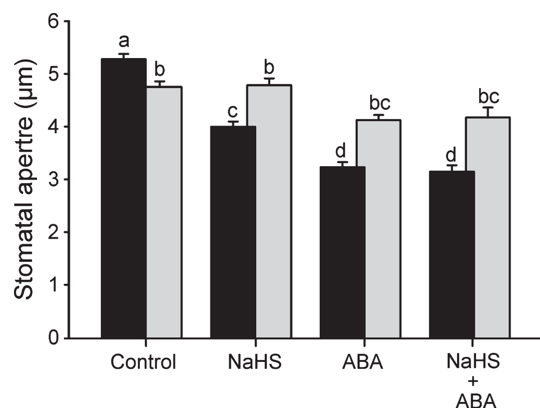

Fig. 3 Effect of the sulphonylurea receptor (SUR) inhibitor glibenclamide (Gli) on abscisic acid (ABA)- and hydrogen sulphide (H_2S)-dependent stomatal closure. *Vicia faba* epidermal strips were preincubated for 2 h in opening buffer (10 mM K-MES, pH 6.1; 10 mM KCl) and then treated for 90 min with 100 µM NaHS or 50 µM ABA in the absence (black bars) or presence (grey bars) of 1 µM Gli. Different letters indicate statistical differences between treatments (Dunn's test, $P < 0.001$).

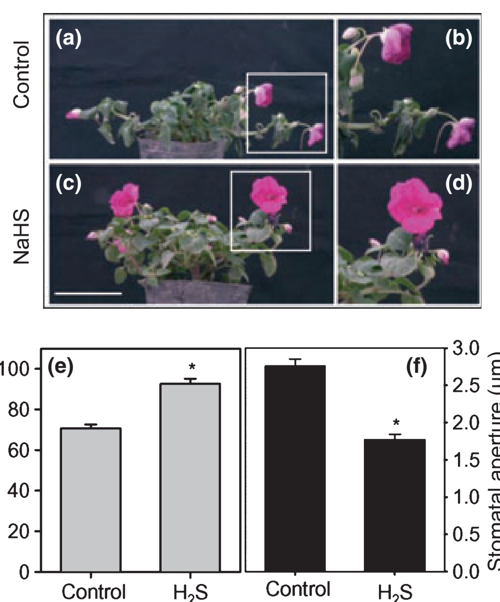

Fig. 4 Hydrogen sulphide (H_2S) protects *Impatiens walleriana* plants from drought stress. (a–d) *I. walleriana* plants were watered with 100 ml of either water (control) or 100 µM of the H_2S donor NaHS and left without watering for 4 d. The pictures were taken on the fourth day after treatment and are representative of three different experiments performed with five pots each. (e) Relative water content (RWC) of *I. walleriana* leaves taken from plants after 4 d without watering. Values are expressed as means ± SE (n = 6). Asterisks denote statistical differences (t-test, $P < 0.001$). (f) Stomatal aperture values of *I. walleriana* epidermal strips preincubated for 2 h in opening buffer and then incubated for 90 min in the absence (control) or presence of 100 µM NaHS (H_2S). Asterisks denote statistical differences with respect to control (0 µM NaHS) (t-test, $P < 0.001$). Values are expressed as means ± SE and represent the mean of 20–30 stomata from at least three independent experiments (n = 100–120). Bar, 5 cm.

Stomatal regulation is strictly related to plant water status. Thus, to study the effects of H_2S at the whole-plant level, we treated *I. walleriana* plants (a species extremely sensitive to soil humidity) with either H_2O or NaHS and suspended the watering for 4 d. After the imposed water stress, control plants showed clear wilting symptoms, while NaHS-treated plants were greener and more turgid (Fig. 4a–d). The macroscopic observations were supported by physiological measurements. Fig. 4(e,f) shows that NaHS treatment resulted in a 20% reduction of water loss compared with the control (represented as RWC) and the induction of stomatal closure. In the same manner, NaHS-treated *V. faba* leaves subjected to desiccation showed RWC values 10% higher than control plants (data not shown). These last results agreed with recently published data showing that H_2S alleviated drought imposed on soybean seedlings (Zhang *et al.*, 2010).

Discussion

Biologically active gases have emerged as key regulator molecules and effectors in a myriad signalling events. It has been proposed that gasotransmitters posses advantages as biologically active compounds (Moore *et al.*, 2003; Mustafa *et al.*, 2009b). While NO and CO have already been shown to participate in different key processes of plant physiology (Cao *et al.*, 2007; Lamattina & Polacco, 2007), H_2S is just emerging. In the present work we show that exogenous H_2S released by H_2S donors induces stomatal closure in different plant species. In addition, we have also shown that the inhibition of endogenous H_2S formation impairs ABA-induced stomatal closure in both *V. faba* and *A. thaliana*. These results agreed with microarray data obtained from the Arabidopsis eFP Browser (Winter *et al.*, 2007), showing a 70% increase in DES1 expression after treating Arabidopsis seedlings with 100 µM ABA for 1 h (data not shown). Even though it was previously reported that atmospheric H_2S, ranging from 0.24 to 0.74 µl l^{-1} H_2S, has little or no effect on spinach, pumpkin or spruce transpiration rates (Kok *et al.*, 1989), this apparent discrepancy may result from either differences in H_2S concentration between intra- and extracellular spaces or from the use of different tissues or plant species. The same issue has been recently reviewed for mammalian systems where tissue concentration of free H_2S is orders of magnitude lower than the H_2S concentrations required to alter tissue functions (Furne *et al.*, 2008). In our laboratory, measurements obtained using a Micro Sulfide Ion Electrode showed that H_2S concentration in leaf extracts from *V. faba* and *A. thaliana* ranges between 1 and 5 µM, values that agreed with those reported for *A. thaliana* leaves (Papenbrock *et al.*, 2007). With the methodology we are using at the moment, we did not find any difference in H_2S concentrations in leaves subjected to

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ABA treatments or PAG. This could be because of the equipment's sensitivity or because differences in H_2S concentrations induced by ABA treatment are restrained to 'hot spots', so the difference is underestimated in whole-cell extracts. The existence of H_2S 'hot spots' or microenvironments within the cell has been suggested by Furne *et al.* (2008), and the identification of cellular and subcellular locations of H_2S production will be important in the future.

Another guard cell second messenger that has excited the attention of plant scientists is hydrogen peroxide (H_2O_2), which was shown to have a key role in ABA-dependent signalling. H_2O_2 exerts its effect through the regulation of different components, such as ion channels and NO formation (Wang & Song, 2008). ABA triggers an increase in endogenous H_2O_2 concentrations during stomatal closure induction (Zhang *et al.*, 2001). Moreover, ABA-dependent stomatal closure is partially blocked in guard cells by scavenging H_2O_2 with catalase or by the inhibition of NADPH oxidase activity (Zhang *et al.*, 2001). Some of the more recent reports on H_2S biology in plants have shown that H_2S counteracts the oxidative burst generated by H_2O_2 production upon different stresses by reducing H_2O_2 concentrations and increasing the activity of antioxidant enzymes (Zhang *et al.*, 2008, 2009a, 2010). As a consequence, it can be argued that H_2S might be preventing H_2O_2 signalling in guard cells. Hence, extracellular addition of H_2S should impair ABA-induced H_2O_2-mediated stomatal closure. However, in this work we present data showing that there are no differences between stomatal aperture values for ABA and ABA + NaHS treatments (Figs 2b, 3). These results could indicate that the concentration of H_2S is enough to scavenge H_2O_2 and partially induce stomatal closure acting downstream of H_2O_2 on H_2S-specific targets. Future studies showing the ABA-dependent production of H_2O_2 in the presence of exogenous addition of H_2S will be needed to address this point. Another interesting aspect is that H_2S induced stomatal closure at incubation times of 80 min or longer, suggesting that H_2S is not an early ABA-signalling event such as H_2O_2 and Ca^{2+} (Pei & Kuchitsu, 2005) and that, as stated earlier, it is probably acting on different targets in guard cells.

Abscisic acid signalling in guard cells has become a model for signal transduction studies in plants. The signalling pathways triggered by ABA in guard cells are so complex that they resemble a scale-free network (Hetherington & Woodward, 2003). Based on a pharmacological approach, we showed that the inhibition of L-Cys desulphydrase activity partially blocks ABA-dependent stomatal closure. This effect is restored by the addition of exogenous H_2S. These results lead us to propose that endogenous H_2S might be acting within the ABA-signalling network, possibly through the regulation of MRPs. Future studies using the AtMRP5 null mutant (Gaedeke *et al.*, 2001) and L-CDES-deficient (*des1-1*, *des1-2*) insertion mutants (Alvarez *et al.*, 2010) will provide a complementary genetic approach to support the data presented in this work.

It has been recently postulated that H_2S is working through a mechanism called sulphydration (Mustafa *et al.*, 2009a). H_2S might be directly acting on Cys residues that contain S–H bonds, converting them into S–S–H and thus modifying protein activities. A recent report demonstrates the central role of extracellular Cys residues in rvSUR1 to activate the K^+-ATP channel by H_2S (Jiang *et al.*, 2009).

Despite the constant advances being made in understanding guard cell signalling mechanisms, new components are still appearing. In this work we present the first evidence showing H_2S participation in ABA regulation of stomatal closure. Further studies are needed to understand and unveil downstream targets of H_2S. This report opens a new window for the study of H_2S in plant signalling, and adds fresh knowledge for the improvement of crop tolerance to drought.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Schematic representation of H_2S enzymatic biosynthesis pathways in plants.

Fig. S2 H_2S induces stomatal closure in *Arabidopsis thaliana*.

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